Reports on the Proficiency Testings for *Trichinella* spp. organised by the EURLP for the NRLs

March, 2014

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A Report of the NRL Proficiency Testing to detect *Trichinella spiralis* larvae in pork and/or horse meat samples according to the EU directive 2075/2005

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A Report of the NRL Proficiency Testing to detect *Trichinella spiralis* larvae in pork and/or horse meat samples according to the EU Directive 2075/2005

1 Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites circulating in most European countries in both wild and domestic animals (Pozio et al., 2009). Humans acquire the infection by the consumption of raw or undercooked meat from pigs, horses, wild boars and other game animals (Pozio et al., 2003). According to the Commission Regulation (EC) No 2075/2005, all animals which are potential carrier of *Trichinella* spp. infective larvae should be tested at the slaughterhouse according to one of the approved test.

2 Scope

One of the core duties of the EURL for Parasites is to organise proficiency testings, as it is stated in the Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The scope of this comparison is to test the competence of the appointed NRLs to detect *Trichinella* larvae in pork and/or horse meat samples according to one of the approved methods reported in the Annex 1 of the Commission Regulation EC No 2075/2005.

3 Proficiency testing organization

For the first time, the proficiency testing (PT) registration, result submission, and reporting, have been organized online on a dedicated section (*restricted area*) of the EURLP web site (http://www.iss.it/crlp/index.php?lang=2&id=53&tipo=10).

4 Time frame

The PT was announced to NRLs by email on 3 February, 2014 and the deadline to make the online registration was February 21, 2014. On March 17, 2014, the samples were dispatched to participants by an international courier. Reporting deadline was April 12, 2014.

5 Test material

The test material forwarded to each laboratory consisted of 3 meat balls made with 100 ± 5 grams (or 35 ± 2 grams) of minced pork or 3 meat balls made with 100 ± 5 grams (or 35 ± 2 grams) of minced horse meat. Two meat balls were spiked with 4 viable *Trichinella spiralis* larvae each and one meat ball did not contain any larva, as negative control.

Larvae were obtained by a partial artificial digestion of *T. spiralis*-infected mice (isolate code ISS003), according to the EURLP SOP. Experimental infection of mice by *Trichinella* spp. muscle stage larvae. Larvae were counted under a stereo-microscope using a watch glass of 2 cm of diameter and transferred to the meatball rinsing the watch glass with PBS. To ensure that no larva remained on the glass, it was examined twice under the stereo-microscope and rinsed with PBS allowing the PBS to reach the core of the ball (see Annex 2).

Each meat ball was close in a plastic bag and under vacuum sealed, a code was added on the envelop. Each envelope containing the meat ball was then stored at +4°C until the forwarding.

The 3 meat balls under vacuum were forwarded in a polystyrene box containing ice packs. In the box, ice packs were separated from the meat samples by a cardboard separator to

avoid a direct contact. The packages were forwarded according to the international forwarding regulations by an international courier.

To check the sample stability over time, and to estimate the suitability of the packing and forwarding conditions under which the meat balls were sent, two groups of 3 meat balls each were packaged as those forwarded, stored at room temperature, and tested at the EURLP three and five days after packaging.

Each participant lab was invited to enter the information about package content and its condition of preservation at opening in the online “shipment form”.

6 Instructions to participants

Instructions were available to all participants at the “instruction” page of the web site.

7 Qualitative evaluation

To successfully pass the PT, each participant had to correctly identify both positives and negatives samples.

8 Quantitative Evaluation

Because of the low number of samples, it was impossible to apply a Z-score or any other statistical parameter. The results were evaluated according to the following criteria:

- the detection of at least 2 larvae was considered acceptable for sample spiked with 4-5 larvae;
- the detection of at least 1 larva was considered acceptable for sample spiked with 1-3 larvae.

Overestimation:

- a maximum overestimation of 2 larvae was considered acceptable for sample spiked with 4-5 larvae;
- a maximum overestimation of 1 larva was considered acceptable for sample spiked with 1-3 larvae.

The difference between expected and observed results was reported to allow the lab to monitor their results overtime and to compare them with the performance of the other labs.

9 Statistical analysis

The analysis of data was performed using the STATA software; the statistical significance of the results was confirmed by the Kruskal-Wallis test.

10 Participating laboratories

Of the 28 MS, Luxembourg appointed the Belgium NRL for parasites and Slovak Republic did not participate, all the other NRLs (#26) participated in the PT. In addition, NRLs from Iceland, The former Yugoslav Republic of Macedonia, Montenegro, Norway, Serbia and Switzerland, agreed to participate. Both the NRLs of Croatia participated, consequently, a total of 33 labs were involved in the PT.

11 Results

11.1 Delivery of the package to NRLs

Out of 32 packages (Italian package not included), 22 were delivered within 24 hs, 7 within 48 hs, 3 within 72 hs, and one was the NRL of Italy located in the same Institute of the EURLP. At delivery, the internal temperature of packages delivered within 48 hs was less than 10°C, whereas for packages delivered within 72 hs the internal temperature was between 10° C and 15 °C. The time elapsed from the arrival of the package at the NRL and the control of its content was within 2 hs for all NRLs.

11.2 Digestion methods

The magnetic stirred method for the pooled sample digestion was the most used (29 NRLs, 87.9%); the mechanically assisted pooled sample digestion method/sedimentation technique was used in 2 NRLs (8%), and the automatic digestion method for pooled samples up to 35 g was used in 2 NRLs (6%).

11.3 Type of meat samples

Out of 33 participating laboratories, 29 labs (87.9%) requested pork samples of 100 ± 5 g, 2 labs (6%) pork samples of 35 ± 2 g, and 2 labs (6%) horse meat samples of 100 ± 5 g.

11.4 Qualitative results

All 33 participating laboratories passed the PT, neither false positive nor false negative samples were reported.

11.5 Quantitative results

Out of 33 participating laboratories, 6 labs (18.1%) did not satisfied the criteria to pass the quantitative evaluation. No participant overestimated the number of larvae spiked in the samples.

11.6 Descriptive statistic of the results

For each laboratory, the difference between the expected and observed number of larvae for each sample, and the mean value, calculated over the three samples, were evaluated (Annex 3). The graphical representation of the mean values obtained by each lab and the overall mean value calculated over all laboratories is shown in Annex 4. The mean values obtained by laboratories in 2007-2014 period, are shown in Annex 5. All labs show an improvement in the digestion test performance from 2007 to 2008, while fluctuating results were observed from 2008 to 2014. The comparison of the overall mean difference (i.e., the mean of relative difference values considering all samples and all laboratories), confirms that a strong improvement was made from the first (2007) to the second (2008) PT, while the improvement is less evident in the further years (Table 1). In 2012, the overall mean difference value increased a little bit comparing to the previous years, but in 2013, the overall mean difference value decreased and in 2014 it reached the lowest value.

Table 1 - Overall mean difference comparison for the 2007-2014 period

Year	2007	2008	2009	2010	2011	2012	2013	**2014**
Relative difference values	0.41	0.25	0.23	0.22	0.20	0.26	0.21	**0.16**

12 Conclusions

The comparison of the 2007-2014 results, shows that there has been an improvement in the laboratory performances from the first (2007) to the second (2008) PT, while during the following years the improvement was less evident. The absence of laboratories reporting a number of larvae greater than the number of larvae spiked into the sample witness an increased accuracy in the count, but it can be also explained by the reduced number of samples and the reduced number of larvae in each sample. This year for the first time, all participant laboratories passed the PT and neither false positive nor false negative samples were reported, moreover the overall mean difference value obtained was lower than that obtained in the previous year, witnessing the high degree of expertise achieved by NRLs in this specific field.

13 References

Pozio E., Rinaldi L., Marucci G., Musella V., Galati F., Cringoli G., Boireau P., La Rosa G. (2009) Hosts and habitats of *Trichinella spiralis* and *Trichinella britovi* in Europe. Int. J. Parasitol. 39:71-79.

Pozio E., Gomez Morales M.A., Dupouy Camet J. (2003). Clinical aspects, diagnosis and treatment of trichinellosis. Expert Review of Anti-infective Therapy 1:471-482.

Commission Regulation (EC) No 2075/2005 of 5 December 2005 laying down specific rules on official controls for Trichinella in meat. OJ L338/60-82.

Commission Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. OJ L191/1-59.

Community Reference Laboratory for Parasites (2007). Guideline for the detection of *Trichinella* larvae at the slaughterhouse or connected laboratory in a Quality Assurance System, pp. 1-14, www.iss.it/crlp/docu/.

B Report of the NRL Proficiency Testing on *Trichinella* spp. larva identification at the species level by a molecular method

1 Introduction

In the MS of the European Union, four species of the genus *Trichinella* are circulating among domestic and/or wild animals: *Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi* and *Trichinella pseudospiralis*. *Trichinella britovi* is the most widespread species infecting prevalently carnivore mammals but also wild boars; whereas, it is seldom detected in domestic pigs. This species is circulating in most of MS excluding Cyprus, Denmark, Ireland, Luxembourg, Malta and UK (Pozio et al., 2009). *Trichinella spiralis* infecting prevalently domestic pigs and wild boars is more scattered in single foci present in Austria, Bulgaria, Finland, Germany, Hungary, Lithuania, Poland, Romania, Ireland and North Ireland (UK), Slovak Rep., Spain and Sweden (Pozio et al., 2009). *Trichinella nativa* is circulating among carnivore mammals living in cold regions (Estonia, Finland, Latvia, Lithuania and Sweden) but recently it has been detected in one red fox of Poland and in three red foxes of Germany. *Trichinella pseudospiralis* is the only species infecting both mammals and birds (Pozio and Zarlenga, 2013). The main reservoir host is the wild boar; seldom this species has been detected in carnivore mammals (foxes and raccoon dogs). This species has been detected in 16 MS (excluding Austria, Belgium, Cyprus, Greece, Ireland, Latvia, Luxembourg, Malta, Portugal, Romania, Slovenia, and Spain). The identification of the *Trichinella* species is extremely important for epidemiological investigation and to trace back the origin of infections in animals and humans. These parasites can be distinguished among them only by molecular methods (Pozio and La Rosa, 2010). Several protocols are today available in the literature, but only few of them allow to identify single organisms (Identification of Trichinella Muscle Stage Larvae at the species level by Multiplex PCR).

2 Scope

According to of the Commission Regulation (EC) No 2075/2005 (Annex 1, Chapter I), when *Trichinella* spp. larvae are detected by digestion of animal muscles, the larvae should be stored in 90% ethyl alcohol and identified at the species level at the EURLP or NRLs. During the NRL Workshop of 2010, participants requested the EURLP to organise a PT on the identification of *Trichinella* spp. larvae at the species level. The scope of this PT is to test the competence of NRLs to correctly identify the species circulating in Europe and those which can be introduced from other continents.

3 Time frame

The proficiency testing (PT) was announced to NRLs by email on 3 February, 2014 and the dead line to send the participation form was February 21, 2014. On March 17, 2014, the samples were dispatched to participants by an international courier. Reporting deadline was April 12, 2014.

4 Participating laboratories

Of the 28 MS, Luxembourg appointed the Belgium NRL for parasites, only 17 MS agreed to participate at the PT (Austria, Belgium, Bulgaria, Croatia, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Netherland, Poland, Romania, Slovak Rep., Slovenia and Spain). In addition, NRL from Iceland, Serbia and Switzerland, agreed to participate (see Annex 1). A total of 20 NRLs participated in the PT.

5 Test material

The test material forwarded to each laboratory consisted of *Trichinella* muscle larvae in ethanol. According to the lab requests, two different type of samples were forwarded: pool of larvae or single larvae. The panel of pooled larvae consisted of four vials containing 10 larvae of *T. spiralis, T. nativa, T. britovi,* and *T. pseudospiralis.* The single larva panel consisted of 12 vials containing one single larva each (i.e., three vials for each of the four species). Larvae were obtained by artificial digestion of mouse carcasses infected with *T. spiralis* (isolate code ISS003), *T. nativa* (isolate code ISS70), *T. britovi* (isolate code ISS002), and *T. pseudospiralis* (isolate code ISS013), according to the EURLP SOP (see paragraph 5). Participating labs were invited to use one of the published methods available in the international literature. Each laboratory received, together with samples, the appropriate form to register the results (Annex 6).

6 Results

6.1 Type of samples

Out of 20 participating laboratories, 12 labs (60%) requested a pool of larvae and 8 labs (40%) requested single larvae.

6.2 Methods of analysis

Out of 20 labs which received PT samples, 14 labs followed the EURLP protocol (two of them made minor modification to the original protocol), 3 labs followed the protocol published by Pozio and La Rosa (2010) (two of them made minor modification to the original protocol), 2 lab followed the protocol published by Zarlenga et al. (1999) (one of them made minor modification to the original protocol), and 1 lab followed both the protocol published by Zarlenga et al. (1999) and the protocol of De Bruyne et al. (2005).

6.3 Molecular identification

6.3.1 PCR products

All labs which analyzed pool of larvae were able to obtain PCR products, while out of six labs which analyzed single larvae, two laboratories (code 6 and 7) failed to obtain PCR products from 1 sample.

6.3.2 Species identification

Independently of the type of sample (single larva or pool of larvae), 5 labs failed to correctly identify some larvae at the species level (Annex 8) (three of them participated for the first time at this PT).

7 Conclusions

Out of 20 labs, 15 (75%) were able to correctly identify all larvae at the species level; of them, 7 labs analyzed pools of larvae and 8 labs single larvae. The reasons for PT failure in 25% of labs can be ascribed to the use of not suitable diagnostic protocol or tools (reagents and apparatuses), and to the lack of experience in multiplex PCR band pattern analysis.

8 References

De Bruyne A, Yera H, Le Guerhier F, Boireau P, Dupouy-Camet J, 2005. Simple species identification of *Trichinella* isolates by amplification and sequencing of the 5S ribosomal DNA intergenic spacer region. Vet Parasitol. 132, 57-61.

Pozio E, Hoberg E, La Rosa G, Zarlenga DS, 2009. Molecular taxonomy, phylogeny and biogeography of nematodes belonging to the *Trichinella* genus. Infect Genet Evol. 9, 606-616.

Pozio E, La Rosa G, 2010. *Trichinella*. In: Dongyou, L. (ed.), Molecular detection of foodborne pathogens. Taylor and Francis, CRC Press, Boca Raton, London, New York, pp. 851-863.

Pozio E, Zarlenga DS, 2013. New pieces of the *Trichinella* puzzle. Int J Parasitol 43, 983-997.

Zarlenga DS, Chute MB, Martin A, Kapel CM, 1999. A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. Int J Parasitol. 29, 1859-1867.

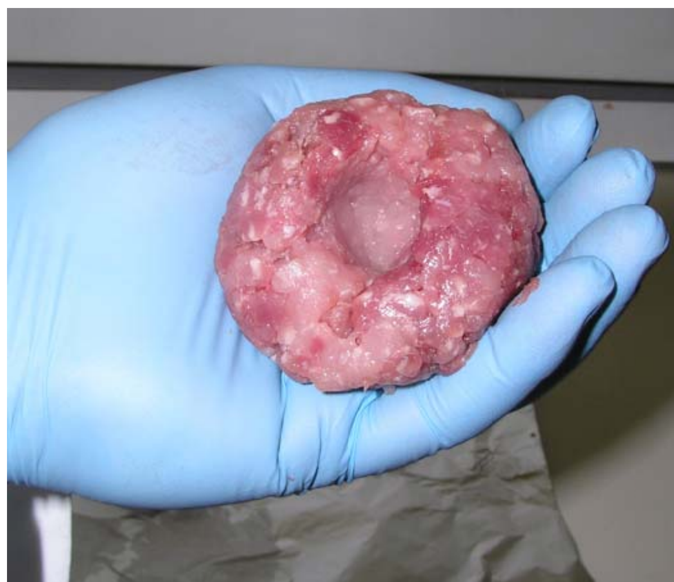
Annex 1

National Reference Laboratories (NRL) participating at the proficiency testings for *Trichinella*

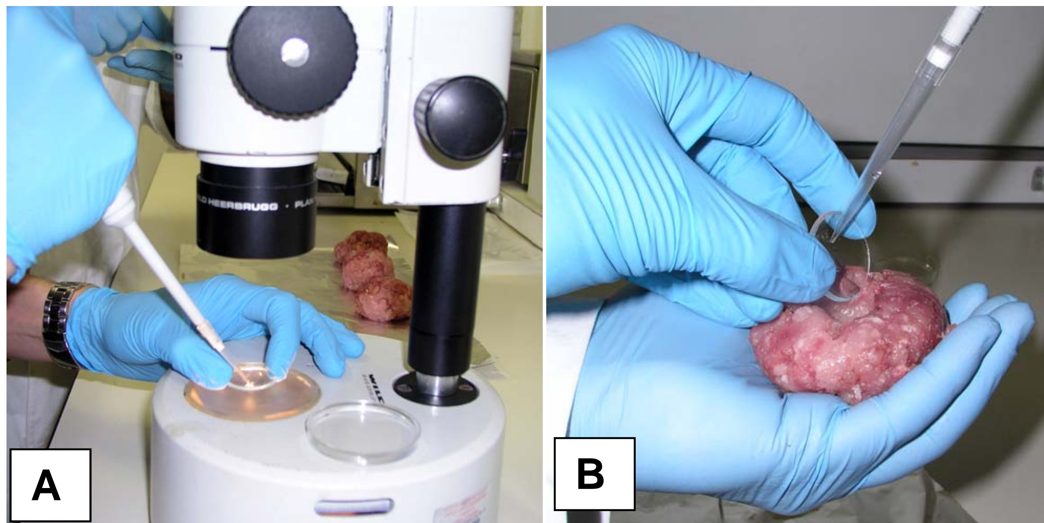
NRL for parasites	Country	PT digestion	PT PCR
Institut für Veterinärmedizin, Innsbruck	Austria	yes	yes
Institute of Tropical Medicine, Antwerp	Belgium	yes	yes
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria	yes	yes
Croatian Veterinary Institute, Zagreb	Croatia	yes	yes
Croatian Veterinary Institute Vinkovci	Croatia	yes	no
State Veterinary Laboratory, Nicosia	Cyprus	yes	no
State Veterinary Institute, Olomouc	Czech Rep	yes	no
National Veterinary Institute, Copenhagen	Denmark	yes	no
Estonian Veterinary and Food Laboratory, Tartu	Estonia	yes	yes
Research Unit Finnish Food Safety Authority, Evira, Oulu	Finland	yes	yes
Food borne parasite NRL & UMR BIPAR ANSES, ENVA, UPEC, ANSES-Laboratoire de Santé Animale, Maison Alfort	France	yes	yes
Federal Institute for Risk Assessment, BfR, Berlin	Germany	yes	yes
Centre of Athens Veterinary Institutions, Athens	Greece	yes	yes
Central Veterinary Institute, Budapest	Hungary	yes	no
Central Meat Control Laboratory, Celbridge, County Kildare,	Ireland	yes	no
Istituto Superiore di Sanità, Rome	Italy	yes	yes
Laboratory of Food and Environmental Investigations, Riga	Latvia	yes	yes
National Food and Veterinary Risk Assessment Institute, Vilnius	Lithuania	yes	no
National Veterinary Laboratory, Albertown, Marsa	Malta	yes	no
National Institute of Public Health and the Environment, Bilthoven	Netherlands	yes	yes
National Veterinary Research Institute, Pulawy	Poland	yes	yes
Laboratório Nacional de Investigação Veterinária, Lisboa	Portugal	yes	no
Hygiene and Public Veterinary Health Institute, Bucharest	Romania	yes	yes
State Veterinary and Food Institute, Bratislava	Slovakia	no	yes
National Veterinary Institute, Ljubljana	Slovenia	yes	yes
Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain	yes	yes
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden	yes	no
Veterinary Laboratories Agency, Weybridge	UK	yes	no
Keldur - Institute for Experimental Pathology, Reykjavík	Iceland	yes	no
Diagnostic veterinary laboratory, Podgorica	Montenegro	yes	no
Norwegian Veterinary institute, Oslo	Norway	yes	no
Institute for Laboratory Diagnostic INEP, Belgrade	Serbia	yes	yes
Department of Parasitology, University of Skopje	FYROM	yes	no
Institute of Parasitology, University of Bern	Switzerland	yes	yes

Annex 2

Meatball samples (35g and 100g)



An hollow is made in the center of each meatball to house the larvae



Larvae are counted under a stereo-microscope using a watch glass (A) and transferred to the meatball rinsing the watch glass with 200 µl of PBS (B)

The watch glass is examined under the stereo-microscope to ensure that no larva remains on it

Each sample is under vacuum sealed (A) and labeled with a numeric code (B)

Annex 3

Descriptive statistics of the absolute difference between expected and reported count by laboratory for all samples (horse meat and pork) in 2014

Laboratory code	No. of samples	Mean	SD
NRL1	3	1	1.73
NRL2	3	0.33	0.58
NRL3	3	0.33	0.58
NRL4	3	0	0
NRL5	3	0	0
NRL6	3	0	0
NRL7	3	0	0
NRL8	3	0	0
NRL9	3	0.67	1.15
NRL10	3	0.33	0.58
NRL11	3	0	0
NRL12	3	1.3	1.53
NRL13	3	0	0
NRL14	3	0.33	0.58
NRL15	3	1	1.74
NRL16	3	0.33	0.58
NRL17	3	0	0
NRL18	3	1.33	1.58
NRL19	3	0.67	1.15
NRL20	3	0.33	0.58
NRL21	3	0.33	0.58
NRL22	3	0.33	0.58
NRL23	3	0	0
NRL24	3	0	0
NRL26	3	1.67	1.53
NRL34	3	1	1
NRL35	3	0	0
NRL40	3	0.33	0.58
NRL41	3	0.67	0.58
NRL42	3	0	0
NRL43	3	1.67	1.53
NRL44	3	0.67	0.58
TLE6	3	0	0
All	99	0.44	0.85

Kruskal-Wallis test, $p = 0.4310$

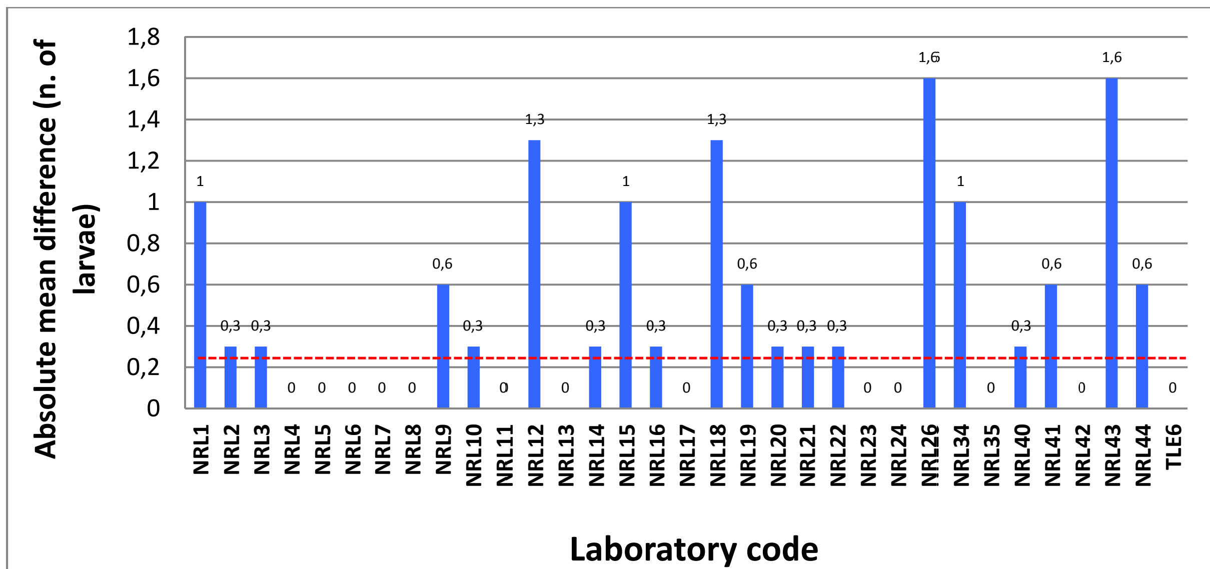
Annex 4

Descriptive statistic of the difference between the expected and reported larval count by laboratory and comparison among the years 2007-2014.

Laboratory code	Mean							
	2007	2008	2009	2010	2011	2012	2013	2014
NRL1	0.36	0.24	0.11	0.25	0.23	0.35	0.16	0.37
NRL2	0.14	0.22	0.07	0.08	0.09	0.08	0	0.12
NRL3	0.52	0.37	0.23	0.10	0.29	0.34	0.16	0.12
NRL4	0.20	0.12	0.03	0.12	0.13	0.13	0	0
NRL5	0.14	0.06	0.08	0.12	0.09	0.26	0.1	0
NRL6	0.30	0.08	0.17	0.10	0.04	0.17	0	0
NRL7	0.23	0.05	0.03	0.07	0.04	0	0	0
NRL8	0.13	0.08	0.03	0.12	0.02	0.05	0.2	0
NRL 9	0.13	0.19	0.06	0.11	0.18	0.23	0.2	0.25
NRL10	0.17	0.45	0.28	0.41	0.15	0.14	0.1	0.12
NRL11	0.33	0.22	0.24	0.23	0.16	0.36	0.36	0
NRL12	0.72	0.10	0.10	0.26	0.18	0.5	0.1	0.5
NRL13	1	0.36	0.40	0.25	0.14	0.1	0.1	0
NRL14	0.75	0.12	0.20	0.12	0.18	0.16	0.36	0.12
NRL15	0.64	0.64	0.42	0.25	0.42	0.63	0.8	0.37
NRL16	0.41	0.33	0.13	0.23	0.33	0.62	1	0.12
NRL17	0.86	0.26	0.68	0.05	0.14	0.09	0	0
NRL18	0.26	0.27	0.45	0.37	0.53	0.34	0.5	0.5
NRL19	0.08	0.12	0.03	0.16	0.08	0.08	0.26	0.25
NRL20	-	0.19	0.08	0.01	0.07	0.03	0	0.12
NRL21	0.40	0.25	0.02	0.51	0.16	0.03	0.1	0.12
NRL22	0.36	0.41	0.30	0.23	0.15	0.18	0.16	0.12
NRL23	0.48	0.38	0.17	0.08	0.26	0.43	0.33	0
NRL24	0.45	0.27	0.37	0.36	0.26	0.24	0.1	0
NRL25	0.70	0.43	0.75	0.26	0.50	0.46	0.16	-
NRL26	-	-	-	0.15	0.11	0.33	0.2	0.62
NRL34	-	-	0.23	0.54	0.23	0.34	0	0.37
NRL35	-	-	-	0.44	0.36	0.59	-	0
NRL40	-	-	-	-	-	0.15	0.16	0.12
NRL41	-	-	-	-	-	-	0.16	0.25
NRL42	-	-	-	-	-	-	-	0
NRL43	-	-	-	-	-	-	-	0.62
NRL44	-	-	-	-	-	-	-	0.25
TLE6	-	-	-	-	-	-	0.6	0

Annex 5

Graphical representation by histograms of the absolute difference between expected and reported count of all samples (horse meat and pork), by laboratory



Annex 6

European Union Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 6A

Laboratory _______

Proficiency test to identify *Trichinella* larvae at the species level by a molecular method

Identification of pool of larvae

Results

Sample code	Molecular Method[1]	Date (dd/mm)	Identified species	Notes

1) Explain briefly or add a reference for the molecular method used.

Date ____________________

Technician (name/sign): ____

viale Regina Elena, 299 – 00161 Rome, Italy
Tel.: +39 06 4990 2304; Fax: +39 06 4990 3561, e-mail: edoardo.pozio@iss.it

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European Union Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 6B

Laboratory _______

Proficiency test to identify *Trichinella* larvae at the species level by a molecular method

Identification of single larvae

Results

Sample code	Molecular Method[1]	Date (dd/mm)	Identified species	Notes

1) Explain briefly or add a reference for the molecular method used.

Date ____________________

Technician (name/sign): ____

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Annex 7

PT on *Trichinella* spp. larva identification at the species level by a molecular method

Lab code	Sample type	Method used	N. correct identification	N. incorrect identification	N. missed identification	Final evaluation
NRL16	pool	Zarlenga et al. 1999*	4	0	0	positive
NRL7	single	EURLP protocol	11	0	1	positive
NRL11	pool	EURLP protocol	4	0	0	positive
NRL44	pool	EURLP protocol	3	1	0	negative
NRL23	pool	EURLP protocol	4	0	0	positive
NRL1	single	EURLP protocol	12	0	0	positive
NRL10	single	EURLP protocol	12	0	0	positive
NRL8	pool	Pozio and La Rosa 2003	4	0	0	positive
NRL22	single	EURLP protocol*	9	3	0	negative
NRL42	pool	EURLP protocol*	3	1	0	negative
NRL4	single	EURLP protocol	12	0	0	positive
NRL3	single	EURLP protocol	12	0	0	positive
NRL6	single	Zarlenga et al. 1999 and 5S	11	0	1	positive

		sequencing				
NRL24	single	EURLP protocol	12	0	0	positive
NRL17	pool	EURLP protocol	4	0	0	positive
NRL34	pool	EURLP protocol	4	0	0	positive
NRL25	pool	EURLP protocol	4	0	0	positive
NRL2	pool	Pozio and La Rosa 2003*	3	1	0	negative
NRL21	pool	Pozio and La Rosa 2003*	4	0	0	positive
NRL35	pool	Zarlenga et al. 1999	2	2	0	negative

*some minor modifications were made to the original protocol